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ABSTRACT

Several investigators in diverse geographical locations have detected airborne mutagens with the Ames' Salmonella typhimurium bioassay. Yet to be established is the aerodynamic diameter of the mutagen-containing particles. To study this matter, an Andersen 2000 sampler was used to collect size-fractionated samples in Durham, NC. The samples were extracted and concentrated, the masses were determined, and the mutagenic activities were bioassayed. Initial studies were designed to establish a protocol suitable for routine air sampling. A sufficient sample quantity was obtained by operating four of the Andersen samplers simultaneously over a 5-7 day interval. The results of 10 weekly samplings indicated that most of the deposited mass, and most of the mutagenic activity, occurred in the fractions containing the small (less than 2 μm) particles. Selected extracts, positive for mutagenicity, were tested for the presence of electrophiles by reaction with nitrobenzylpyridine (a chemical nucleophile) or by reaction with electrons provided in the course of dc polarographic analysis. These chemical tests verified the presence of direct-acting alkylating agents in extracts of deposited aerosols.

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CONTENTS

Abstract iii
Figures vi
Tables vi

1. Introduction 1
2. Conclusions 2
3. Materials and Methods 3
4. Experimental Procedures. 5
5. Discussion 12

References 14

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FIGURES

Number		Page
1	Polarographic reduction of air pollution XAD fractions	8
2	Nitrobenzyl pyridine reactivity of air pollution extract . . .	11

TABLES

1	Mass of Pollutants Deposited and Extracted	9
2	Mass Distribution of Organic Extracts (μg extract/m^3)	9
3	Particle Size Distribution of TA98 Mutagenicity.	10
4	Effect on S-9 on TA98 Mutagenicity	10

SECTION 1

INTRODUCTION

The discharge of combusted organic matter into the air has long been a process of concern to environmental scientists and others involved in pollution control. Chemical, physical and biological consequences associated with the generation, aerosolization, and dispersion of waste matter throughout the troposphere are subjects that concern scientists in many disciplines. These challenging problems attract the attentions of diverse workers, leading to interdisciplinary communication and new problem-solving approaches.

This report is the result of a collaborative effort between our laboratory and the Environmental Sciences Research Laboratory. We were charged with the task of developing a method that could be used to investigate the particle size distribution of airborne alkylating agents detected as mutagens. Our strategy was to deposit the size-fractionated aerosol particles on non-reactive surfaces (coated aluminum foil or Teflon-coated glass fiber filters) where they could be readily extracted for bioassay.

SECTION 2

CONCLUSIONS

The Ames' Salmonella typhimurium bioassay was useful for detecting airborne alkylating agents in size-fractionated aerosols, provided that enough deposit was collected on each stage of the sampler. The Ames' TA98 strain was most useful since it was more sensitive than other test strains to the activities present in the collected samples.

All weekly samplings at a site in Durham, N.C., yielded one or more fractions that were positive in the Ames' test. Consistently, fractions containing particles $\leq$2 microns were positive. These fractions contained about 72% of the total collected mass and 80% of the total mutagenic activity.

The alkylating agents detected as mutagens did not require metabolic activation by liver enzymes. Samples also contained substances that reacted positively in a colorimetric test for alkylating agents and appeared to be reducible by polarographic titration.

SECTION 3

MATERIALS AND METHODS

SAMPLING SITE

All fractions tested in this study were collected from a site located on the roof of the Durham, EPA Test Site, Mangum and Cora Streets, Durham, N.C. This building is located approximately 300 m north of the Durham expressway and 250 m east of the American Tobacco Company plant.

SAMPLING PROTOCOL

Andersen 2000C samplers (1,2) were used to separate airborne particulate matter into five aerodynamic diameter fractions. Stages 1,2,3, and 4 collected particles from 7-11 μm, 4.7-7 μm, 3.3-4.7 μm, and 2.1-3.3 μm, respectively. The backup filters collected smaller particles.

The initial months of the study were used to establish a sampling procedure to provide sufficient deposits for mutagenesis testing. Matters of concern included the quality of air, mass distribution of pollutants, length of the sampling period, and treatment of filters. From the initial data, we concluded that approximately 20,000 M^3 of filtered air would deposit enough mass for the bioassay of each size category. This sample size was attained by operating four Andersen 2000 samplers in parallel over a 5-7 day period. Each sampler was loaded with four coated aluminum foil filters on stages 1-4 and a backup filter below stage 4. Samplers were operated at 20 ft^3/min over the sampling period. The filters were then carefully removed and packaged for shipping to Riverside. Ten samples were collected and tested between June 15 and November 3, 1978.

Three types of filters were used as backup filters: Gelman glass fiber filters; Teflon-coated glass fiber filters; and Teflon supported by polypropylene fibers. The choice of filter did not affect the bioassay

results, but the teflon treated glass fiber filters tended to clog and were discontinued on October 24.

Originally, the aluminum foil filters were coated with polypropylene glycol, but this substance proved to be insufficiently adherent; some of the coating was extracted, precluding the determination of pollutant mass. This problem was alleviated by replacing polypropylene glycol with propylene glycol as the coating beginning with the August 22 sampling.

SAMPLE PREPARATION

Aluminum foil filters from corresponding stages of the four samplers were pooled. Areas of the filters bearing deposit were removed with scissors and placed in Soxhlet extraction thimbles. Backup filters were cut into small strips and placed in thimbles. Loaded thimbles were placed in Soxhlet extraction tubes and extracted for six hours with 100 ml acetone (Mallinckrodt AR grade). The extracts were then concentrated *in vacuo* and their residues were transferred to preweighed vials, dried with nitrogen, and weighed.

Stock solutions suitable for bioassay were prepared by adding sufficient dimethyl sulfoxide (DMSO, Aldrich gold label) to make the final concentrations 20,000 "cubic meter equivalents" (cme)/ml in the cases of the foil extracts and 2,000 cme/ml in the case of the backup filter extracts (one "cubic meter equivalent" is defined as the quantity of acetone-extractable residue derived from one 1 m^3 of samples air).

SECTION 4

EXPERIMENTAL PROCEDURES

BIOASSAY

Ames' Salmonella typhimurium bioassay (3) was used to measure the mutagenic activities in the extracts from foil and backup filters. In the case of foil filters, sufficient extract was unavailable for testing in several strains of bacteria. Consequently, initial screenings were executed to determine the most responsive strains; which proved to be TA98, verifying previous reports on air pollution mutagenicity testing (4-8).

Four doses of each extract are tested and the results are expressed as net TA98 revertants/500 cme/plate. This mutagenicity test detects the presence of direct-acting frameshift mutagens in the deposited aerosols in the various size-fractionated samples.

Extracts obtained from the ten weekly samplings were bioassayed for TA98 mutagenicity via an Ames' soft agar test (3). The responsiveness of the strain was checked with the positive control, benzo(a)pyrene. Each sample was bioassayed within two weeks of collection. Foil filter extracts were tested at 250, 500, 1000, and 2000 cme/plate by adding the appropriate amount of extract (in 0.1 ml DMSO) to tubes containing 2.5 ml top agar and the tester strain, TA98 (0.1 ml of fully grown culture). Top agar mixtures were plated and incubated 48 hours prior to the counting of revertant colonies. The background count of spontaneous revertants was measured in each set of experiments: For each test plate, net revertants equalled gross revertants minus spontaneous revertants. For comparison, the results are expressed in units of net revertant counts/500 cme; 500 cme proved to be the highest non-toxic dosage.

Backup filter extracts were similarly tested, except that the dosages used were 25, 50, 100, and 200 cme/plate, and the bioassays were conducted

in the presence and absence of liver S-9. Lower dosages were necessary to avoid toxicity to the tester strain. The liver S-9 was included as a test for metabolic requirements. The results, reported in net revertants/500 cme, were calculated by extrapolating from values obtained in the testable dosage range (i.e., 25-200 cme/plate).

CHEMICAL TESTS

Preussmann et al. demonstrated that a wide variety of alkylating agents can be detected with nitrobenzylpyridine as the nucleophile (10). The nitrobenzylpyridine test for alkylating agents was adapted for use in testing air samples. A backup filter extract from the October 17-24 sampling was tested at several concentrations using the procedures described below.

The desired amount of sample in 1 ml acetone was mixed with 0.5 ml of aqueous potassium acid phthalate (0.1 N) and 0.5 ml nitrobenzylpyridine reagent (5% w/v in acetone); a boiling chip was added and the mixture was held at 105-110°C for 45 min; allowed to cool to 40-50°C and diluted to 4.5 ml with 50% aqueous acetone. The chromophore was generated by additions of potassium carbonate (1M, 0.5 ml) and was extracted into 5 ml of methylene chloride. Absorbance was read from a spectrophotometer at 560 nm.

In another chemical test, an active fraction from the August 22 backup filter extract was analyzed by differential pulse polarography. This analytical technique has been used for the determination of metal ions and electroactive organic molecules, including the nitrosamines. The use of polarography in the determination of a substance involves either its reduction at the cathode or its oxidation at the anode. In a typical assay, an increasing potential is applied to a mercury indicator electrode immersed in a cell containing the species to be reduced. The current remains nearly constant until the applied potential is sufficient to reduce the substituent, at which point a sharp increase in current is sensed by the galvanomenter. The current continues to increase with voltage until the flow of electrons is limited by the diffusion of the reducible species to the mercury cathode. The magnitude of the limiting current, the diffusion current, is proportional to the concentration of the species.

A preliminary experiment was conducted to examine the feasibility of polarography in analyzing air pollution extract fractions. XAD-Chromatography

was employed to separate the extract into nonpolar (active) and polar (inactive) fractions (See fig. 1). A Princeton Applied Research Corporation Model 174 polarographic analyzer, connected to a Hewlett-Packard XY recorder and a PARC Model 303 drop former was used to investigate the electroactivity of the XAD fractions. Titrations were done in a cell containing 10 ml of acetonitrile, 80 µg of the test substance, and tetrabutylammonium perchlorate as the supporting electrolyte. The current was recorded over the voltage range of -0.5V-1.0V.

SUMMARY OF METHODOLOGY

We have combined the technologies of aerosol fractionation and mutagenicity testing to develop a method to determine the particle size distribution of airborne alkylating agents. Our current procedure is summarized below.

Four Andersen 2000 samplers are loaded with aluminum foil filters on stages 1-4 to collect particles 2-11 µm in aerodynamic diameter. For smaller particles, a backup filter (glass fiber or Teflon-supported by polypropylene fibers) is inserted in each sampler below stage 4.

The four samplers are placed at the sampling site and operated at 20 cfm for 168 hours.

Stage 1 filters are pooled, areas of aerosol deposition are excised, and total samples is extracted for 6 hours with acetone in a Soxhlet apparatus. Stage 2,3, and 4 backup filters are similarly processed.

Acetone extracts are evaporated to dryness under vacuum and the residues are dissolved in DMSO to make stock solutions of 20,000 cme/ml (stages 1-4) or 2000 cme/ml (backup filters).

The extracts are tested for mutagenicity with the Ames' Salmonella typhimurium strain, TA98.

We have now completed the testing of 10 weekly samples collected from the Durham, N.C., site. The calculations presented below suggest refinements in our methods. These refinements are postulated in section 5.

Calculations

To arrive at an indication of air quality for each weekly sampling, the quantity of matter deposited on backup filters was divided by the volume of

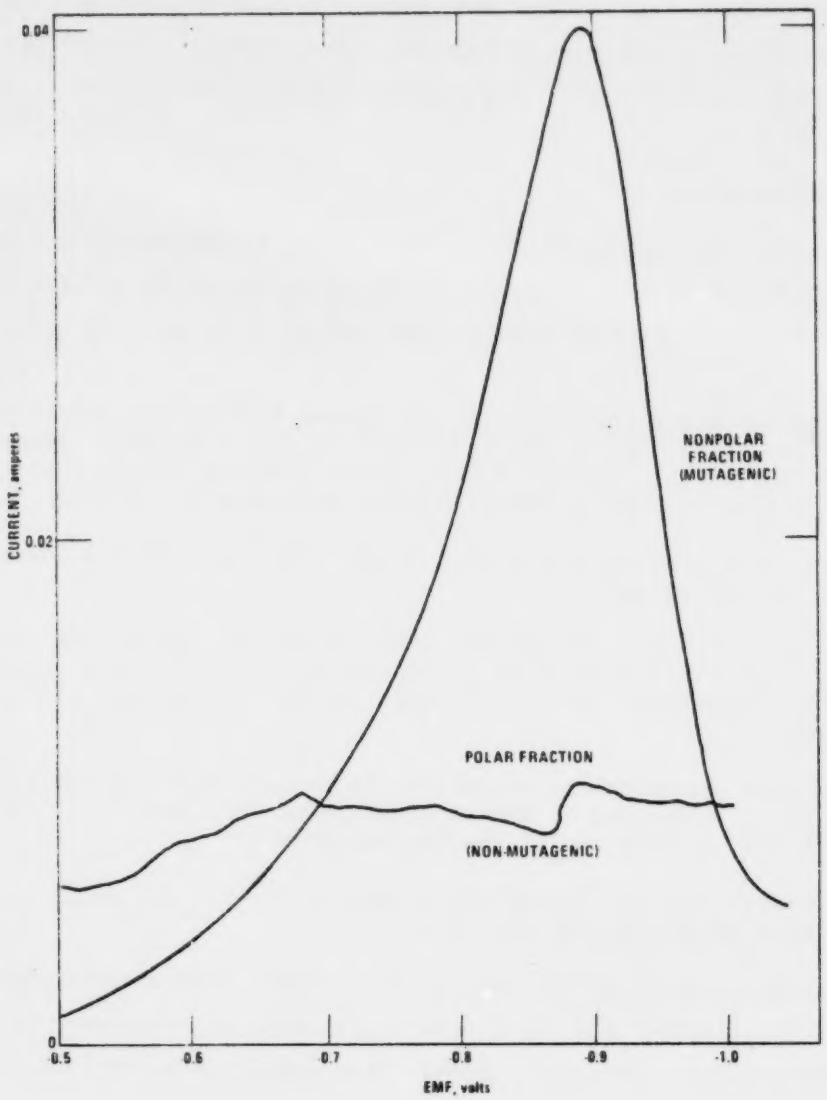

Figure 1. Polarographic reduction of air pollution XAD fractions.

air sampled. This value averaged 28.4 $\mu g/m^3$ and, on the average, 45% of this mass was extracted by acetone (Table 1).

TABLE 1. MASS OF POLLUTANTS DEPOSITED AND EXTRACTED

Week	μg deposit/m^3	μg extract/m^3	μg extract/ μg deposit
6-15	26	9.4	.36
6-28	31	8.1	.26
7-11	21	9.4	.45
8-1	12	9.2	.83
8-22	38	14.5	.38
9-13	29	12.2	.42
9-26	24	12.0	.51
10-5	24	11.2	.47
10-17	45	17.6	.39
10-27	34	15.9	.47
	$\bar{\mu}$ = 28.4±8.8	11.9±0.9	0.45±0.045

The assessment of airborne particulate mass distribution commenced with the August 22-29 samples. Results (Table 2) indicate that approximately 72% of the total extractable mass is derived from particles collected on the backup filters (<2 microns). The distribution of mutagenic activity appeared to parallel the distribution of mass, as most of the activity was detected in extracts from the smallest-sized particles (Table 3). The correlation coefficient (r) for mass distribution versus activity distribution was 0.76.

TABLE 2. MASS DISTRIBUTION OF ORGANIC EXTRACTS (μg extract/m^3)

Week	Stage I	Stage II	Stage III	Stage IV	Backup
8-22	.6	0.45	0.5	1.5	14.5
9-13	1.1	0.75	0.8	6.6	12.2
9-26	3.0	3.28	3.2	2.26	12.0
10-5	0.2	0.41	0.25	0.06	11.2
10-17	1.1	1.2	0.46	1.6	17.6
10-27	0.7	0.77	0.49	0.81	15.9
	$\bar{\mu}$ = 1.12±0.36	1.14±0.39	0.95±0.41	2.13±0.86	13.9±0.94

TABLE 3. PARTICLE SIZE DISTRIBUTION OF TA98 MUTAGENICITY

Week	TA98 revertants/500 cme/plate				
	1 (7-11μ)	2 (4.7-7μ)	3 (3.3-4.7μ)	4 (2.1-3.3μ)	Backup (<2μ)
6-15/6-22	227	562	59	62	975
6-28/7-5	160	46	0	172	655
7-11/7-18	11	1	0	0	675
8-1/8-9	74	64	14	50	545
8-22/8-29	124	54	13	42	703
9-13/9-19	34	54	60	74	683
9-26/10-3	0	6	4	6	855
10-5/10-13	0	11	25	16	462
10-17/10-24	57	91	0	156	2677
10-27/11-3	33	72	59	101	2887
$\bar{x}$ =	72±23	96±50	23.4±8	68±18	1111±267

The inclusion of liver S-9 in the bioassay did not enhance the mutagenicity of the backup filter extracts, except in the case of the June 15-22 sample (Table 4). In most cases, liver S-9 inclusion slightly reduced the mutagenic activities.

TABLE 4. EFFECT OF S-9 ON TA98 MUTAGENICITY

Week	TA98 revertants/500 cme/plate	
	liver S-9 excluded	liver S-9 included
6-15	975	3139
6-28	655	601
7-11	675	296
8-1	545	578
8-22	703	612
9-13	683	544
9-26	855	626
10-5	462	398
10-17	2677	1937
10-27	2887	1697
	1111±267	1042±276

One of the extracts (10-17 10-24) was tested for the presence of alkylating agents that react with 4-nitrobenzylpyridine. A positive concentration-dependent response was obtained (Figure 2).

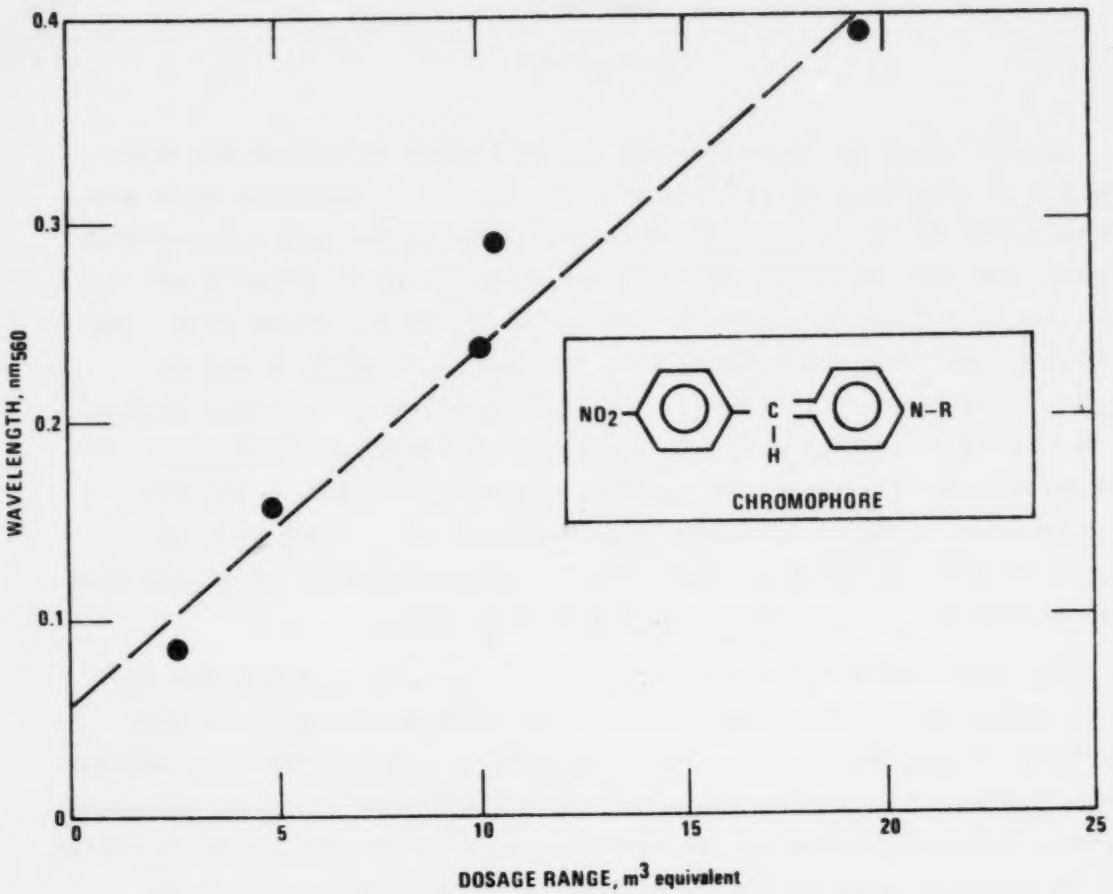

Figure 2. Nitrobenzyl pyridine reactivity of air pollution extract.

SECTION 5

DISCUSSION

Several years ago Bruce Ames and his colleagues recognized the mutagenicity of complex mixtures of organic matter such as cigarette smoke and air pollution extract. Since these initial observations were made, several reports have been published regarding the mutagenicity of polluted air collected on various localities in the United States and Europe (4-9). It appears evident that the production of atmospheric mutagens is not an atypical phenomenon, but rather a widespread occurrence. Although origins of the active substances cannot be pinpointed, a striking correlation between atmospheric lead and mutagenicity has been reported, along with positive data on the mutagenicity of auto exhaust (9). These data, of course, do not rule out the possibility that stationary sources of pollution also contributed to the atmospheric burden of mutagens.

The first objective of this report was to describe a method that may prove useful for investigating whether or not mutagens occur on airborne particles of respirable size. The method involves three phases: a sampling phase; a sample preparation phase; and a bioassay phase. Sampling utilizes a system designed to simulate the respiratory system in that larger particles are deposited on upper stages and smaller particles penetrate to deeper stages. Collected samples are then extracted with acetone which is removed *in vacuo* and the residue is transferred and weighed. DMSO stock solutions are prepared and bioassayed with the Ames' test (strain TA98).

The second objective was to present data that have thus far been obtained via the collection-and-testing system described above. Conclusions concerning the health effects of air pollution mutagenicity are not to be drawn from these data, although the results may provide incentive for further research.

A refinement in the bioassay procedure can be made in view of the current results that establish an effective dosage range (100-500 cme/plate for foil filters, 25-200 cme/plate for backup filters). Samples should be tested at several doses within this range and activity reported as the slope of the best-fit line.

Another modification in the testing phase would be to routinely include assays for chemical electrophiles. Nitrobenzylpyridine assay and polarographic analysis may be the appropriate means. A chemical test for airborne alkylating agents would be a valuable tool for monitoring air quality.

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